

**The immune response of bovine mammary epithelial cells to live or heat-inactivated
*Mycoplasma bovis***

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25 **Abstract**

26 *Mycoplasma bovis* is an emerging bacterial agent causing bovine mastitis. Although these cell wall-
27 free bacteria lack classical virulence factors, they are able to activate the immune system of the
28 host. However, effects on the bovine mammary immune system are not yet well characterized and
29 detailed knowledge would improve the prevention and therapy of mycoplasmal mastitis. The aim of
30 this study was to investigate the immunogenic effects of *M. bovis* on the mammary gland in an
31 established primary bovine mammary epithelial cell (bMEC) culture system. Primary bMEC of four
32 different cows were challenged with live and heat-inactivated *M. bovis* strain JF4278 isolated from
33 acute bovine mastitis, as well as with the type strain PG45. The immune response was evaluated 6
34 and 24 h after mycoplasmal challenge by measuring the relative mRNA expression of selected
35 immune factors by quantitative PCR. *M. bovis* triggered an immune response in bMEC, reflected by
36 the upregulation of tumor necrosis factor- α , interleukin(IL)-1 β , IL-6, IL-8, lactoferrin, Toll-like
37 receptor-2, RANTES, and serum amyloid A mRNA. Interestingly, this cellular reaction was only
38 observed in response to live, but not to heat-inactivated *M. bovis*, in contrast to other bacterial
39 pathogens of mastitis such as *Staphylococcus aureus*. This study provides evidence that bMEC
40 exhibit a strong inflammatory reaction in response to live *M. bovis*. The lack of a cellular response
41 to heat-inactivated *M. bovis* supports the current hypothesis that mycoplasmas activate the immune
42 system through secreted secondary metabolites.

43

44 **Keywords:** Bovine mammary epithelial cells, mastitis, *Mycoplasma bovis*, innate immune response

45 **Introduction**

46 Mycoplasmas represent the smallest self-replicating microorganisms characterized by a minimal
47 genome, and by the lack of a cell wall (Razin et al., 1998). In dairy cattle, *Mycoplasma bovis*
48 belongs to the most prevalent contagious mastitis pathogens (Olde Riekerink et al., 2006) that is
49 often unresponsive to antibiotics and tends to cause chronic infections (Kauf et al., 2007).

50 In general, bacterial species and strain specific severities of mastitis are characterized by differences
51 in their induction of the mammary immune system, i.e. the regulation of several immune factors
52 (i.e. cytokines) and recognition receptors (i.e. toll-like receptors; TLR) (Wellnitz and Bruckmaier,
53 2012; Zbinden et al., 2014). *M. bovis* displays a broad range of immunogenic and
54 immunosuppressive characteristics that determine its pathogenicity. The extensive variation of cell
55 surface antigens, also known as variable surface proteins (Vsps), enables the pathogen to evade the
56 host immunity which contributes to the persistent nature of *M. bovis* infections (Razin et al., 1998;
57 Buchenau et al., 2010). Among these Vsps, lipoproteins represent the most abundant fraction (Razin
58 et al., 1998). Besides their antigenic properties, lipoproteins play a central role in adhesion and are
59 expected to trigger the production of pro-inflammatory cytokines (Pilo et al., 2007). Furthermore,
60 *Mycoplasma*-derived secondary metabolites have been demonstrated to exert cytotoxic effects in
61 infected host cells (Pilo et al., 2005).

62 As *M. bovis* resists antibiotic therapies, it is of high importance to understand the bovine
63 mammary immune response to this pathogen to optimize prevention and therapy strategies of
64 mycoplasmal mastitis in dairy cows. Therefore, the objective of the present study was to investigate
65 the innate immune response of bovine mammary epithelial cells (bMEC) to *M. bovis*, characterized
66 by the mRNA expression of selected immune factors. Primary cultures of bMEC challenged with
67 live and heat-inactivated *M. bovis* were used to investigate host-pathogen interactions that trigger
68 the immune reaction in the mammary epithelium.

69

70 **Materials and Methods**

71 *M. bovis* strains

72 The field strain JF4278, isolated from mastitic milk in 2008 in Switzerland (Aebi et al., 2012), and
73 the type strain PG45 (Hale et al., 1962) were used in this study. *M. bovis* cultures were grown in
74 SP4 broth medium supplemented with 50 µg/ml cefoxitin sodium salt (Sigma-Aldrich, Buchs,
75 Switzerland) for 18 hours (Freundt, 1983).

76 To standardize the assays, standard growth curves relating turbidity (OD₆₀₀) of liquid cultures to
77 colony forming units (CFU) of *M. bovis* were obtained by determining colony counts on SP4 agar
78 supplemented with 50 µg/ml cefoxitin sodium salt incubated for 3-4 days at 37 °C in a humidified
79 atmosphere. Growth of *M. bovis* was tested to assess variations among each individual SP4 batch.
80 Each culture was centrifuged for 30 min at 400 g and at 4 °C. The pellet was suspended in
81 challenge medium consisting of DMEM/F12 supplemented with 5 % FBS, and ITS containing 0.5
82 mg/ml insulin, 0.5 mg/ml apo-transferrin, 0.5 µg/ml sodium selenite (all components were received
83 from Sigma-Aldrich, Buchs, Switzerland) to reach the appropriate concentration. To evaluate the
84 effective mycoplasmal concentrations, serial 10-fold dilutions of *M. bovis* cultures were
85 simultaneously plated on SP4 agar for colony enumeration. For heat-inactivation, *M. bovis*
86 suspensions were incubated for 30 min at 95 °C. The efficiency of this method was previously
87 assessed by plating serial 10-fold dilutions of the *M. bovis* suspension on SP4 agar plates.

88

89 *Treatment of bMEC with live or heat-inactivated M. bovis*

90 Primary cultures of mammary gland epithelial cells of four lactating Holstein dairy cows with
91 clinically healthy udders (somatic cell count <10⁵ cells/ml) were performed as previously described
92 (Wellnitz and Kerr, 2004). Mammary tissue was removed directly after slaughter with permission
93 of the slaughterhouse Marmy SA, Estavayer-le-Lac, Switzerland. Cells were passaged twice and

cryopreserved in DMEM/F12 containing 20 % FBS and 10 % DMSO (Sigma-Aldrich, Buchs, Switzerland) and stored in aliquots at -80°C until the experiment.

Exogenous *Mycoplasma* sp. contamination of cell cultures from all four cows was excluded using the VenorTMGeM Mycoplasma Detection Kit (Minerva Biolabs, Berlin, Germany) following the manufacturer's instructions.

Cells from all cows were thawed and separately cultured in growth medium (DMEM/F12 supplemented with 10 % FBS, penicillin G [500 units, Sigma-Aldrich], streptomycin [100 $\mu\text{g/ml}$, Sigma-Aldrich], and ITS). After two passages, cells were seeded at a concentration of 3×10^5 cells/well on BD FalconTM 6-well cell culture plates (BD Biosciences, San Jose, CA, USA). On the following day, growth medium was replaced with medium without antibiotics.

Assuming that 3×10^5 cells/well after 24 h of incubation and a confluence of about 70 % represent approximately 1×10^6 cells/well, bMEC were challenged with an expected multiplicity of infection (MOI) of 150 of live, or heat-inactivated *M. bovis* suspensions. As a positive control, cells were challenged with 150 MOI of heat-inactivated *S. aureus* strain 1904 from bovine mastitis (Zbinden et al., 2014). Cells incubated in growth medium only served as negative controls.

Cells were grown and incubated at 37°C with 5 % CO_2 .

Total RNA extraction and quantitative real-time PCR

After 6 and 24 h of incubation cells were harvested with 1 ml peqGOLD TrifastTM (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Total RNA was extracted according to the manufacturer's protocol. Reverse transcription with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega Corp., Madison, WI, USA) and quantitative real-time PCR analysis using the Sensimix DNA Kit (Quantace, Biolabo, Châtel St. Denis, Switzerland) on a Rotor-Gene 6000 (Corbett Research, Sydney, Australia) was described before (Zbinden et al., 2014). Primer sequences and annealing temperatures are shown in table 1.

119 Cycle threshold (Ct) values were determined by the Rotor-Gene software version 1.7.75, and the
120 relative mRNA expression was calculated using the comparative Ct method using the following
121 equation (Schmittgen and Livak, 2008): $\Delta Ct = Ct \text{ target gene} - Ct \text{ endogenous control}$ (arithmetic
122 mean of housekeeping genes).

123 To visualize the impact of *M. bovis*, or *S. aureus* on the immune response of bMEC, data are
124 presented as $\Delta\Delta Ct \pm SEM$, where: $\Delta\Delta Ct = \Delta Ct \text{ sample (6, or 24 h; treated)} - \Delta Ct \text{ negative control}$
125 (6, or 24 h; untreated).

126

127 *Statistical Analysis*

128 Data from the cells of four cows were expressed as means \pm SEM from $\Delta\Delta Ct$ values of the selected
129 factors. Statistical analysis of the inflammatory response data was tested for significance ($P < 0.05$)
130 by ANOVA using PROC MIXED of SAS (Release 9.4; SAS Institute Inc., Cary, NC, USA). The
131 model included time, strain, and their interaction as fixed effects, and the cow as repeated subject. A
132 Bonferroni correction was applied.

133

134 **Results**

135 *Cell cultures*

136 Primary bMEC cultures of all four cows grew to an approximate confluence of 70% within ~20 h
137 after seeding into 6-well plates. According to Venor™GeM Mycoplasma Detection Kit, the cultures
138 of all cows were free of *Mycoplasma sp.* contamination. After 24 h of incubation with live or heat-
139 inactivated mycoplasmas, cells were nearly confluent, and no morphological changes were visible.

140

141 *Growth of M. bovis strains*

142 For both *M. bovis* strains a MOI of 150 was expected based on the assumption/observation that an
143 OD_{600} value of 0.1 corresponded to 4.2×10^8 CFU/ml and 1.65×10^9 CFU/ml, for JF4278 and

PG45, respectively. The plating of serial dilutions indicated that the growth kinetics differed between *M. bovis* strains, resulting in effective MOIs of 108 (JF4278), and 30 (PG45), respectively. Plating of heat-inactivated *M. bovis* did not result in any bacterial growth.

Relative mRNA expression of immune factors

The mRNA expression of measured immune factors was not increased in negative controls.

In cells challenged with *S. aureus* 1904 as positive control, an expected induction of mRNA expression of interleukin (IL)-1 β , tumor-necrosis factor (TNF)- α , IL-8, serum amyloid A (SAA), lactoferrin (Lf), and TLR2 after 6 h and 24 h of stimulation was detectable. The chemokine RANTES (regulated on activation, normal T-cell expressed and secreted) was induced at 6 h, but not at 24 h after challenge. IL-6, IL-10, cyclooxygenase (COX)-2, TLR1, and TLR6 were not expressed at the mRNA level at either of the time points in response to *S. aureus*.

Induction of mRNA expression after challenge and differences between challenges with live and heat-inactivated *M. bovis* are shown in Table 2 and Figure 1. Transcription of IL-1 β , TNF- α , and IL-8 significantly increased ($P<0.05$) in bMEC infected with live *M. bovis* at both, 6 and 24 h post infection. The abundance of IL-6 mRNA was increased in response to JF4278 after 24 h. RANTES was only induced at 6 h post infection in bMEC infected by live JF4278 whereas SAA was upregulated in bMEC at 24 h post infection with both live *M. bovis* strains. Lactoferrin mRNA expression was upregulated in bMEC 6 h after infection with live PG45, whereas transcription of Lf was increased 24 h after infection with both live *M. bovis* strains. Expression of TLR2 mRNA was transiently induced after 6 h in response to live JF4278, and at 24 h post infection in response to live PG45. Interleukin-10, COX-2, TLR1, and TLR6 were not induced in bMEC by either live or heat-inactivated *M. bovis*. With heat-inactivated *M. bovis*, only IL-8 mRNA was upregulated after 6 h of challenge by JF4278. A further increase of mRNA expression from 6 to 24 h of challenge was only detectable for SAA and Lf with live mycoplasmas.

169

170 **Discussion**

171 Cryopreserved primary bMEC cultures in a low passage were previously shown to
172 immunologically respond to different bacteria that play a role in mastitis development (Wellnitz and
173 Kerr, 2004). The cells responded to the stimulation with *M. bovis* by increased transcription of
174 genes relevant in mammary gland innate immunity, which confirms the suitability of this model to
175 investigate the immune response to mycoplasmas. As all cell cultures were free of *Mycoplasma* sp.
176 contamination, a cellular response induced by other *Mycoplasma* species than *M. bovis* was
177 excluded. The biological reproducibility was provided by using cells from four different cows.
178 Different periods of challenge (6 and 24 h) were used to investigate immune factor expression in
179 earlier and later phases of the response. The *S. aureus* strain 1904 was used as a positive control
180 (Zbinden et al., 2014) and proved responsiveness of the cultures used.

181 *M. bovis* strain JF4278 used in the present study represents a Swiss field strain that was isolated
182 from severe mastitis in 2008, when *M. bovis* mastitis outbreaks started to emerge in Switzerland
183 (Aebi et al., 2012). In parallel, bMEC were challenged with the type strain PG45 that was isolated in
184 1961 in the US (Hale et al., 1962). Both *M. bovis* strains induced an immune response in bMEC in
185 the present study; however, a direct quantitative comparison is not possible since effective MOIs
186 were not identical due to differential growth. Although Wellnitz et al. (2006) found that an
187 increasing MOIs of *E. coli* or *S. aureus* did not necessarily result in a stronger immune response in
188 bMEC, an effect of the bacterial cell concentration used for infection/challenge can be possible.

189 An experimental mammary *M. bovis* infection increased the pro-inflammatory cytokines TNF- α
190 and IL-1 β in milk (Kauf et al., 2007). This was reflected by the transcriptional upregulation of IL-
191 1 β and TNF- α in bMEC in the present study. The cytokine IL-6 seems to be involved at later stages
192 of the inflammatory response of bMEC to *M. bovis*, since live *M. bovis* JF4278 induced IL-6
193 transcription not until 24 h after stimulation.

194 Chemokines like IL-8 or RANTES mediate the influx of neutrophils from the blood to the site of
195 infection, reflected by an increase of milk somatic cell count during mastitis. In bMEC IL-8 mRNA
196 expression was increased by *M. bovis* which is in agreement with increased milk IL-8
197 concentrations *M. bovis* mastitis (Kauf et al., 2007).

198 Acute phase protein synthesis also reflects an immune response. In this study, SAA expression
199 significantly raised 24 h after infection with live *M. bovis* but not with heat inactivated
200 mycoplasmas. Since SAA is known to act as a chemoattractant, pathogens that upregulate SAA
201 transcription are likely to be associated with an increase of somatic cell count in the mammary
202 gland, which could influence the progress of the mastitis (Badolato et al., 1994).

203 The enzyme COX-2 is involved in prostaglandin synthesis that is upregulated in pulmonary
204 epithelial cells from pigs by *Mycoplasma hyopneumoniae* (Andrada et al., 2014). However, in the
205 present study COX-2 was not upregulated in bMEC in response to either of the *M. bovis* strains.

206 Lactoferrin is an antibacterial protein that is produced by mammary epithelial cells during
207 clinical bovine mastitis (Wellnitz and Bruckmaier, 2012). Live *M. bovis* induced Lf transcription in
208 the present study. The earlier induction of Lf transcription of strain PG45 compared to JF4278
209 despite the lower MOI could indicate that Lf expression follows a strain-dependent mechanism.
210 Although it was reported that Lf production increases over time in bMEC cultures, likely due to the
211 increasing cell density (Wellnitz and Kerr, 2004), the increased transcription of Lf was not due to
212 this effect since control cells did not express increased levels of Lf mRNA during the time of the
213 experiment.

214 Toll-like receptors, i.e. TLR2 in combination with TLR1 or TLR6, on the surface of host cells
215 recognize lipopeptides within the cell membranes of mycoplasmas (Omuetti et al., 2005). The slight
216 increase of TLR2 transcription in bMEC after infection with *M. bovis* shows that TLR2 is involved
217 in *M. bovis* recognition by mammary epithelial cells. In contrast, transcription of TLR1, and TLR6
218 was not affected.

Mycoplasmal infections are not necessarily associated with a strong inflammatory response and can proceed without apparent clinical symptoms (Chambaud et al., 1999). In the present study the immune suppressive cytokine IL-10 was not upregulated in bMEC by *M. bovis*, which is consistent with the results of Lahouassa et al. (2007). An increase of IL-10 in milk after experimental infection of cows with *M. bovis* shown by Kauf et al. (2007) could be derived from other immune cells.

Most interestingly, in contrast to other mastitis pathogens like *E. coli* or *S. aureus* (Griesbeck-Zilch et al., 2008; Zbinden et al., 2014), bMEC responded only to live, but not to heat-inactivated *M. bovis*. For *Mycoplasma mycoides* subsp. *mycoides*, it was previously shown that heat-inactivation results in impaired cytotoxicity (Dedieu et al., 2005). The main reason for this difference is that cell wall containing bacteria have components like lipoteichoic acid, peptidoglycans, or lipopolysaccharide that are recognized by the host immune system, whereas wall-less mycoplasmas do not have these classical virulence factors. Therefore, mycoplasmas exhibit alternative mechanisms that induce an immune response in bMEC, which are apparently antagonized by heat treatment. Putative *M. bovis*-specific immunogenic factors include cell surface molecules such as lipoproteins that are recognized by bMEC via TLRs. However, the most important role in the activation of an immune response seem to play secreted metabolites that are associated with cell damage and, as a consequence, inflammation and disease (Pilo et al., 2007).

In conclusion, the present study indicates that an infection with *M. bovis* induces a considerable immune response in bMEC shown by increased transcription of several immune factors. Remarkably, a substantial immune response only occurs if bMEC are infected with live but not with heat-inactivated *M. bovis*, indicating a role of released metabolites in the activation of the mammary immune response.

Acknowledgments

243 We thank Sibylle Bürki, of the Institute for Veterinary Bacteriology, Vetsuisse Faculty University
244 of Bern, Switzerland, for mycoplasma preparation. The *Staphylococcus aureus* strain 1904 was
245 kindly provided by Prof. R. Stephan, Institute for Food Safety and Hygiene, Vetsuisse Faculty
246 University of Zurich, Switzerland.

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249 **Conflict of interests**

250 The authors have declared that no competing interests exist.

251

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253 **References**

- 254 Aebi, M., Bodmer, M., Frey, J., Pilo, P., 2012. Herd-specific strains of *Mycoplasma bovis* in
255 outbreaks of mycoplasmal mastitis and pneumonia. *Vet Microbiol* 157, 363-368.
- 256 Andrada, M., Quesada-Canales, O., Suárez-Bonnet, A., Paz-Sánchez, Y., Espinosa de Los
257 Monteros, A., Rodríguez, F., 2014. Cyclooxygenase-2 expression in pigs infected
258 experimentally with *Mycoplasma hyopneumoniae*. *J Comp Pathol* 151, 271-276.
- 259 Badolato, R., Wang, J.M., Murphy, W.J., Lloyd, A.R., Michiel, D.F., Bausserman, L.L., Kelvin,
260 D.J., Oppenheim, J.J., 1994. Serum amyloid A is a chemoattractant: induction of migration,
261 adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes. *J Exp*
262 *Med* 180, 203-209.
- 263 Buchenau, I., Poumarat, F., Le Grand, D., Linkner, H., Rosengarten, R., Hewicker-Trautwein, M.,
264 2010. Expression of *Mycoplasma bovis* variable surface membrane proteins in the
265 respiratory tract of calves after experimental infection with a clonal variant of *Mycoplasma*
266 *bovis* type strain PG45. *Res Vet Sci* 89, 223-229.
- 267 Chambaud, I., Wróblewski, H., Blanchard, A., 1999. Interactions between mycoplasma lipoproteins
268 and the host immune system. *Trends Microbiol* 7, 493-499.
- 269 Davies, D., Meade, K.G., Herath, S., Eckersall, P.D., Gonzalez, D., White, J.O., Conlan, R.S.,
270 O'Farrelly, C., Sheldon, I.M., 2008. Toll-like receptor and antimicrobial peptide expression
271 in the bovine endometrium. *Reprod Biol Endocrinol* 6, 53.
- 272 Dedieu, L., Chapey, E., Balcer-Rodrigues, V., 2005. *Mycoplasma mycoides* ssp. *mycoides* biotype
273 small colony-secreted components induce apoptotic cell death in bovine leucocytes. *Scand J*
274 *Immunol* 62, 528-538.
- 275 Dreesen, L., Rinaldi, M., Chiers, K., Li, R., Geurden, T., Van den Broeck, W., Goddeeris, B.,
276 Vercruysse, J., Claerebout, E., Geldhof, P., 2012. Microarray analysis of the intestinal host
277 response in *Giardia duodenalis* assemblage E infected calves. *PLoS One* 7, e40985.
- 278 Freundt, E.A., 1983. Culture media for classic mycoplasmas. In: Razin, S. and Tully, J.G. (Eds),
279 *Methods in mycoplasmology*. Academic Press, New York, pp. 127-135.
- 280 Griesbeck-Zilch, B., Meyer, H.H., Kühn, C.H., Schwerin, M., Wellnitz, O., 2008. *Staphylococcus*
281 *aureus* and *Escherichia coli* cause deviating expression profiles of cytokines and lactoferrin
282 messenger ribonucleic acid in mammary epithelial cells. *J Dairy Sci* 91, 2215-2224.
- 283 Hale, H.H., Helmboldt, C.F., Plastringe, W.N., Stula, E.F., 1962. Bovine mastitis caused by a
284 *Mycoplasma* species. *Cornell Vet* 52, 582-591.
- 285 Kauf, A.C., Rosenbusch, R.F., Paape, M.J., Bannerman, D.D., 2007. Innate immune response to
286 intramammary *Mycoplasma bovis* infection. *J Dairy Sci* 90, 3336-3348.

287 Lahouassa, H., Moussay, E., Rainard, P., Riollot, C., 2007. Differential cytokine and chemokine
288 responses of bovine mammary epithelial cells to *Staphylococcus aureus* and *Escherichia*
289 *coli*. *Cytokine* 38, 12-21.

290 Mukesh, M., Bionaz, M., Graugnard, D.E., Drackley, J.K., Looor, J.J., 2010. Adipose tissue depots
291 of Holstein cows are immune responsive: inflammatory gene expression in vitro. *Domest*
292 *Anim Endocrinol* 38, 168-178.

293 Olde Riekerink, R.G., Barkema, H.W., Veenstra, S., Poole, D.E., Dingwell, R.T., Keefe, G.P.,
294 2006. Prevalence of contagious mastitis pathogens in bulk tank milk in Prince Edward
295 Island. *Can Vet J* 47, 567-572.

296 Omuetti, K.O., Beyer, J.M., Johnson, C.M., Lyle, E.A., Tapping, R.I., 2005. Domain exchange
297 between human toll-like receptors 1 and 6 reveals a region required for lipopeptide
298 discrimination. *J Biol Chem* 280, 36616-36625.

299 Pilo, P., Frey, J., Vilei, E.M., 2007. Molecular mechanisms of pathogenicity of *Mycoplasma*
300 *mycoides* subsp. *mycoides* SC. *Vet J* 174, 513-521.

301 Pilo, P., Vilei, E.M., Peterhans, E., Bonvin-Klotz, L., Stoffel, M.H., Dobbelaere, D., Frey, J., 2005.
302 A metabolic enzyme as a primary virulence factor of *Mycoplasma mycoides* subsp.
303 *mycoides* small colony. *J Bacteriol* 187, 6824-6831.

304 Razin, S., Yogev, D., Naot, Y., 1998. Molecular biology and pathogenicity of mycoplasmas.
305 *Microbiol Mol Biol Rev* 62, 1094-1156.

306 Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T)
307 method. *Nat Protoc* 3, 1101-1108.

308 Wellnitz, O., Bruckmaier, R.M., 2012. The innate immune response of the bovine mammary gland
309 to bacterial infection. *Vet J* 192, 148-152.

310 Wellnitz, O., Kerr, D.E., 2004. Cryopreserved bovine mammary cells to model epithelial response
311 to infection. *Vet Immunol Immunopathol* 101, 191-202.

312 Wellnitz, O., Reith, P., Haas, S.C., Meyer, H.H.D., 2006. Immune relevant gene expression of
313 mammary epithelial cells and their influence on leukocyte chemotaxis in response to
314 different mastitis pathogens. *Vet Med (Praha)* 51, 125-132.

315 Zarrin, M., Wellnitz, O., van Dorland, H.A., Bruckmaier, R.M., 2014. Induced hyperketonemia
316 affects the mammary immune response during lipopolysaccharide challenge in dairy cows. *J*
317 *Dairy Sci* 97, 330-339.

318 Zbinden, C., Stephan, R., Johler, S., Borel, N., Bünter, J., Bruckmaier, R.M., Wellnitz, O., 2014.
319 The inflammatory response of primary bovine mammary epithelial cells to *Staphylococcus*
320 *aureus* strains is linked to the bacterial phenotype. *PLoS One* 9, e87374.

321

322 **Table 1.** Sequences, accession numbers, annealing temperature of the PCR primers, and length of
 323 the PCR products.

Gene ^a	Sequence 5'→3'		GenBank accession no.	Annealing temperature (°C)	Length (bp)
TNF- α	for	CCA CGT TGT AGC CGA CAT C ^b	NM173966	60	155
	rev	CCC TGA AGA GGA CCT GTG AG ^b			
IL-1 β	for	AGT GCC TAC GCA CAT GTC TTC ^b	M37211	60	114
	rev	TGC GTC ACA CAG AAA CTC GTC ^b			
IL-6	for	CTT CAC AAG CGC CTT CAC TC ^b	NM173923.2	62	132
	rev	GTC AGA AGT AGT CTG CCT GG ^c			
IL-8	for	ATG ACT TCC AAG CTG GCT GTT G ^b	AF232704	60	149
	rev	TTG ATA AAT TTG GGG TGG AAA G ^b			
RANTES	for	GCC AAC CCA GAG AAG AAG TG ^b	BC102064	60	119
	rev	CTG CTT AGG ACA AGA GCG AGA ^b			
SAA	for	GGG CAT CAT TTT CTG CTT CCT ^d	AF540564	60	106
	rev	TTG GTA AGC TCT CCA CAT GTC TTT AG ^d			
COX-2	for	TCC TGA AAC CCA CTC CCA ACA ^e	NM174445	62	242
	rev	TGG GCA GTC ATC AGG CAC AG ^e			
Lf	for	GGC CTT TGC CTT GGA ATG TAT ^e	L08604	62	338
	rev	ATT TAG CCA CAG CTC CCT GGA G ^e			
TLR1	for	ACT TGG AAT TCC TTC TTC ACG A ^f	NM001046504	60	176
	rev	GGA AGA CTG AAC ACA TCA TGG A ^f			
TLR2	for	GGT TTT AAG GCA GAA TCG TTT G ^f	NM174197	60	190
	rev	AAG GCA CTG GGT TAA ACT GTG T ^f			
TLR6	for	CCT TGT TTT TCA CCC AAA TAG C ^f	NM001001159	60	154
	rev	TAA GGT TGG TCC TCC AGT GAG T ^f			

IL-10	for	TGT TGA CCC AGT CTC TGC TG ^g	NM174088.1	60	94
	rev	GGC ATC ACC TCT TCC AGG TA ^g			
GAPDH	for	GTC TTC ACT ACC ATG GAG AAG G ^b	NM001034034	60	197
	rev	TCA TGG ATG ACC TTG GCC AG ^b			
Ubiquitin	for	AGA TCC AGG ATA AGG AAG GCA T ^b	NM174133	62	198
	rev	GCT CCA CCT CCA GGG TGA T ^b			

324 for = forward, rev = reverse.

325 ^aRANTES = regulated on activation, normal T cell expressed and secreted; SAA = serum amyloid
326 A; COX-2 = cyclooxygenase-2; Lf = lactoferrin.

327 Primer sequence references : ^b(Griesbeck-Zilch et al., 2008); ^c(Zarrin et al., 2014), ^d(Mukesh et al.,
328 2010), ^e(Zbinden et al., 2014), ^f(Davies et al., 2008), ^g(Dreesen et al., 2012).

329

330 **Table 2.** Changes of mRNA abundance (Mean \pm SEM $\Delta\Delta\text{Ct}^c$) of immune factors in bMEC
 331 challenged with 2 strains of live or heat-inactivated (h.i.) *M. bovis* for 6 or 24 h.
 332

Gene ^d	Time (h)	JF4278		PG45	
		live	h.i.	live	h.i.
TNF- α	6	4.5 \pm 0.9 ^{a*}	1.8 \pm 0.8 ^b	4.9 \pm 0.4 ^{A*}	0.4 \pm 1.0 ^B
	24	4.9 \pm 0.4 ^{a*}	-0.3 \pm 1.0 ^b	4.4 \pm 0.5 ^{A*}	0.5 \pm 0.4 ^B
IL-1 β	6	7.9 \pm 1.0 ^{a*}	1.6 \pm 1.1 ^b	7.9 \pm 1.1 ^{A*}	-0.2 \pm 0.2 ^B
	24	8.1 \pm 0.7 ^{a*}	0.2 \pm 0.5 ^b	9.2 \pm 0.5 ^{A*}	-0.1 \pm 0.4 ^B
IL-6	6	0.9 \pm 2.2 ^a	0.5 \pm 0.3 ^a	1.8 \pm 0.6 ^A	0.0 \pm 0.6 ^A
	24	2.1 \pm 0.4 ^{a*}	-0.1 \pm 0.1 ^a	2.2 \pm 1.0 ^A	-0.2 \pm 0.6 ^A
IL-8	6	5.6 \pm 0.9 ^{a*}	1.1 \pm 0.3 ^{b*}	5.6 \pm 0.3 ^{A*}	0.3 \pm 0.3 ^B
	24	7.1 \pm 1.1 ^{a*}	1.3 \pm 1.1 ^b	7.6 \pm 1.0 ^{A*}	0.1 \pm 1.3 ^B
RANTES	6	1.3 \pm 0.4 ^{a*}	1.0 \pm 0.8 ^a	0.3 \pm 0.4 ^A	-1.2 \pm 0.9 ^A
	24	1.3 \pm 0.6 ^a	-0.7 \pm 0.9 ^a	1.6 \pm 0.8 ^A	-0.5 \pm 0.6 ^A
SAA	6	3.1 \pm 1.8 ^a	0.4 \pm 1.2 ^a	2.9 \pm 1.6 ^A	-0.3 \pm 1.8 ^A
	24	8.5 \pm 0.7 ^{a*#}	-0.6 \pm 0.4 ^b	7.7 \pm 0.1 ^{A*#}	-1.1 \pm 0.7 ^B
COX-2	6	0.6 \pm 0.8 ^a	0.7 \pm 1.2 ^a	1.4 \pm 1.0 ^A	0.1 \pm 0.9 ^A
	24	0.5 \pm 1.5 ^a	0.1 \pm 0.6 ^a	3.0 \pm 1.2 ^A	2.2 \pm 1.1 ^A
Lf	6	0.1 \pm 0.3 ^a	-0.4 \pm 0.3 ^a	1.1 \pm 0.2 ^{A*}	-1.4 \pm 0.3 ^B
	24	4.4 \pm 1.0 ^{a*#}	0.3 \pm 0.7 ^b	4.0 \pm 1.4 ^{A*#}	-1.3 \pm 0.1 ^B
TLR1	6	0.6 \pm 0.5 ^a	0.0 \pm 1.0 ^a	0.7 \pm 0.2 ^A	0.3 \pm 2.0 ^A
	24	-1.6 \pm 0.7 ^a	-0.4 \pm 0.3 ^a	-0.8 \pm 0.4 ^A	-0.6 \pm 0.8 ^A

TLR2	6	$1.9 \pm 0.3^{a*}$	0.2 ± 0.7^b	1.1 ± 1.0^A	-0.4 ± 0.8^A
	24	1.2 ± 0.9^a	0.4 ± 0.6^a	$2.3 \pm 0.7^{A*}$	1.0 ± 0.5^A
TLR6	6	0.1 ± 0.5^a	-0.3 ± 0.2^a	0.0 ± 0.7^A	0.0 ± 0.9^A
	24	-1.3 ± 0.6^a	-0.9 ± 0.3^a	-0.5 ± 0.5^A	0.2 ± 0.4^A
IL-10	6	1.4 ± 1.6^a	0.7 ± 1.3^a	0.9 ± 1.0^A	0.4 ± 0.4^A
	24	-2.8 ± 0.6^a	0.0 ± 0.4^a	-2.3 ± 0.8^A	0.3 ± 0.5^A

333 *Means differ significantly from negative control.

334 #Means between 6 h and 24 h treatment differ significantly.

335 ^{a, b}Means between live and heat-inactivated JF4278 without common superscript letters differ
336 ($P < 0.05$).

337 ^{A, B}Means between live and heat-inactivated PG45 without common superscript letters differ
338 ($P < 0.05$).

339 ^c $\Delta\Delta C_t$ values are normalized to negative controls and corrected for the two reference genes,
340 GAPDH and ubiquitin.

341 ^dRANTES = regulated on activation, normal T cell expressed and secreted; SAA = serum amyloid
342 A; COX-2 = cyclooxygenase-2; Lf = lactoferrin.

343

344

345 **Figure. 1. Changes of TNF- α relative mRNA abundance as a representative for several other**
346 **immune factors in bMEC challenged with *M. bovis* JF4278.**

347 Changes of relative mRNA abundance ($\Delta\Delta C_t$) of TNF- α in bMEC challenged for 6 h, or 24 h with
348 live or heat-inactivated (h.i.) *M. bovis* strain JF4278 (108 MOI). Data are presented as Means \pm
349 SEM.

350 ^{a,b,A,B}Means within time point between live and heat-inactivated JF4278 without common
351 superscript letters differ ($P < 0.05$).

352 *Means differ from negative control ($P < 0.05$).

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